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DEVELOPMENT OF BRASSICA JUNCEA GERMPLASM WITH SEED OIL LOW IN SATURATED FATTY ACIDS

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Development of *Brassica juncea* germplasm with seed oil low in saturated fatty acids.

Project 19990352

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Final Report

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A. ABSTRACT

Researchers have been working on developing *B. juncea* germplasm with an oil profile similar to canola quality *B. napus*. The industry requires that the total saturate level be lower than 7 % to remain competitive in relation to other oilseeds currently on the market. This is a high priority for the Canadian canola industry.

B. juncea is currently grown as a condiment crop on the prairies and modifications to the fatty acid profile are important for the future development of this crop. The objective of this project was to develop canola quality low saturate lines of *B. juncea* via microspore mutagenesis using ethyl methane sulfonate (EMS). Development of a low saturate *B. juncea* variety would meet supply and demand of this commodity.

Microspore mutagenesis techniques have been applied to other canola species such as *B. napus* and *B. rapa* [Ferrie, 2001, 1998] to modify the fatty acid profiles. Over the last three years, there were 142 microspore mutagenesis experiments conducted on *B. juncea* using EMS as the mutagen. From these experiments, 9100 double haploid plants were produced and seed from 2530 mutagenized lines were planted in field trials in collaboration with Agriculture and Agri-food Canada (AAFC) in Saskatoon.

Fatty acid analysis from the 2002 field trials showed saturate levels ranged from 5.41-9.54 of the 330 double haploid lines analysed. The *B. napus* check variety AC Excel had a saturate level of 6.67 and the *B. rapa* check variety AC Parkland was 5.42. Both canola varieties were within the range expected for the saturate levels. There were six *B. juncea* double haploids with fatty acid profiles of interest (i.e. low saturate levels). Further field evaluations will be required to confirm these results. Fatty acid analysis from the 2003 field trials have not yet been performed

B. TECHNICAL REPORT

1. Background

Canola oil is an important source of edible oil and is low in saturated fats, which is important for optimal health and wellness. Nutritionally, unsaturated oleic (18:1), linoleic (18:2) and linolenic acid (18:3) can help lower cholesterol levels. A low saturate *B. juncea* canola would be beneficial to consumers for health concerns.

Agronomically, *B. juncea* has desirable characteristics, which include higher yielding cultivars, resistance to blackleg, resistance to seed shattering, early maturing and seedling vigour. *B. juncea* is also drought tolerant which would make it suitable for growing this species in the hot dry conditions often experienced across the prairie provinces. Modifications to the fatty acid profile of *B. juncea*, could lead to significant advances in the value and competitiveness of canola in relation to other oilseeds currently on the market.

2. Project Objectives

The objectives for this project were:

a. Develop a microspore mutagenesis system for *B. juncea* and to screen for lines with lower saturate levels.
b. Produce 5000-10000 double haploid lines with altered fatty acid profiles.
c. Distribute germplasm into Canadian canola industry.

3. Results and Discussion

a) Donor Plants

Two breeding lines, coded 6893 and 6717 chosen from G. Rakow's *B. juncea* breeding program at Agriculture and Agri-food Canada (AAFC) Research Center in Saskatoon were grown as donor plants for microspore mutagenesis experiments. The total level of saturates for lines 6893 and 6717 are 6.5 and 7.1 respectively.

The donor plants were grown in Conviron growth cabinets (20°/15° C, 16 hour photoperiod) and just prior to bolting the temperature was lowered to 10°/5° C. The plants were watered three times per week with 0.35g/L of 15-15-18 (N-P-K) fertilizer.

b) Microspore culture

i) Basic protocol

A wide range of *B. juncea* cultivars were grown initially to determine optimal conditions for microspore culture. The cultivars grown were Cutlass, Donskja, Blaze, Krisna, Lethbridge, Commercial Brown, Pal 918, Pal947 and Zem 83.

Preliminary microspore culture experiments were conducted on the commercial cultivars and the two *B. juncea* breeding lines (6893 and 6717) from AAFC to determine optimal conditions for microspore embryogenesis for this species.

Microspores were isolated from bud sizes 2-4 mm in length [Hiramatasu, 1995], washed three times in B5-13 [Gamborg, 1968] and cultured in basal medium NLN [Lichter, 1982] media supplemented with 17% sucrose (NLN-17), with or without 0.1 % BA (6-benzylaminopurine), and with or without glutamine [Ferrie, 1995]. Cultures were then incubated in the dark at 32°C or 35°C from 48 hours to 72 hours. In addition, media changes were also conducted after induction using NLN-13 and NLN-10 [Ferrie, 1995; 1992] before moving the cultures to a 24°C incubator for the remainder of 3 weeks.

From the various experiments conducted, results indicated that using NLN-17 with 0.1 % BA without glutamine and the media changed to NLN-13 after the required induction period produced the best embryos in terms of quality and the number of embryos produced. However, the two breeding lines required further optimization, as genotypic differences were observed between experiments.

ii) Chromosome doubling

Colchicine is routinely used in the microspore induction media as an effective and efficient *in vitro* method of chromosome doubling [Ferrie, 1998; Hansen, 1996]. This eliminates the need to apply a colchicine treatment at the plantlet stage and is considered a safer method of application. Colchicine was added to the induction media at a concentration of 10^{-4} M for either 48 hours or 72 hours, (depending on the genotype) and then removed in a media change.

Additional experiments were also conducted using trifluralin as an *in vitro* doubling agent (Zhao, 1995). A concentration of 0.5 uM was tested for both genotypes. There was good induction during the initial incubation period of the cultures, but the induced cells failed to divide further. Results obtained were inconsistent. As a result, routine mutagenesis experiments were conducted using colchicine as the doubling agent.

iii) Determination of embryogenic frequency

The embryogenic frequency was determined for the commercial cultivars and the two breeding lines 6893 and 6717 following the conditions outlined in the basic protocol. Genotypic differences were observed in the embryogenic response among the different lines and between experiments.

Krisna was the most responsive commercial cultivar, producing an average of 686 embryos/100 buds. While all commercial cultivars produced embryos, a range of embryogenesis was observed (Table 1).

Table 1: The frequency of microspore embryogenesis of several *B. juncea* varieties.

Genotype	**Embryogenesis (embryos/100 buds)**	
	Average	**Range**
Krisna	686	320-1233
Cutlass	513	130-1117
Pal 947	178	3-516
Pal 918	186	18-510
Donskja	211	20-467
Com. Brown	37	8-103
Blaze	25	3-46
Lethbridge	22	5-38
Zem 83	9	7-11

The addition of colchicine to the induction media (10^{-4} M) was also evaluated for microspore embryogenic frequencies of the two breeding lines, 6893 and 6717 and was found to have a slight detrimental effect (Table 2). However, embryos were still produced at an efficient rate and therefore, colchicine was routinely used.

Table 2: The frequency of microspore embryogenesis of lines 6717 and 6893.

Genotype	**Embryogenesis (embryos/100 buds)**	
	No colchicine Average (range)	**With colchicine Average (range)**
6717	277 (0-8666)	361 (0-3167)
6893	744 (0-7000)	389 (0-1307)

iv) Microspore density

Embryogenic frequencies for the *Brassica* species can be influenced by the densities at which microspores are cultured [Huang, 1990]. Generally, for *B. napus* cultivars, microspores are cultured at densities between 75, 000 and 100, 000 microspores/ml, [Fletcher, 1998] while *B. rapa* and *B. juncea* cultivars are cultured at a density of 100, 000 microspores/ml [Ferrie, 1995]. Routine experiments were conducted using the recommended density of 100,000 microspores/ml.

c) Microspore mutagenesis

i) Mutagenesis treatment conditions

Microspore mutagenesis experiments were performed following the *B. rapa* protocol developed during the Canola Council of Canada/IRAP *B. rapa* consortium project [Ferrie, 1998] and the conditions outlined in the basic protocol. Isolated microspores from the two breeding lines were treated with Ethyl methane sulfonate (EMS) for 1.5 hours. The EMS was then removed by centrifugation and the microspores were then cultured according to the basic protocol reported.

ii) Killing curve

Experiments were conducted to determine the EMS concentration at which 50 % and 80 % of the microspores were killed for both breeding lines using 0.1% to 0.5 % EMS. The controls received no EMS (Figure 1 and Figure 2). There was a 50 % kill of microspores at 0.1 % EMS and an 85 % kill at 0.2 % EMS for genotype 6893.

There was an increase in embryogenesis using 0.2 % and 0.3 % EMS for genotype 6717 (Figure 1 and Figure 2). This effect has been observed in other *Brassica* species. (A. Ferrie unpublished data). A 60 % kill of microspores was observed at 0.4 % EMS and a 30 % kill at 0.5 % EMS for this genotype.

Figure 1. Effect of EMS on microspore embryogenesis of *B. juncea* lines 6717 and 6893

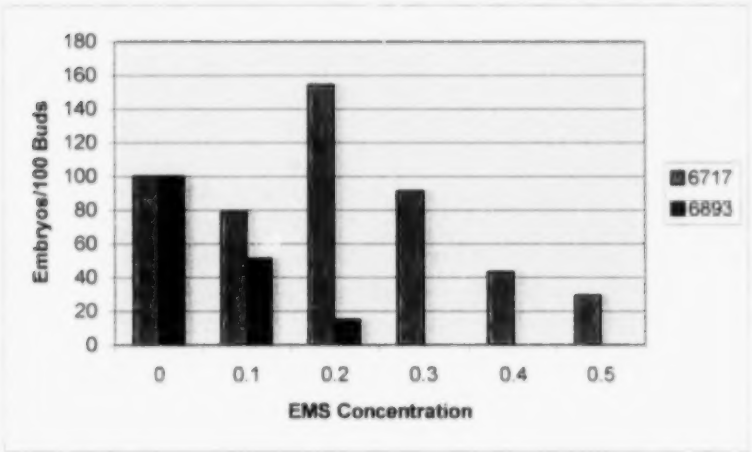
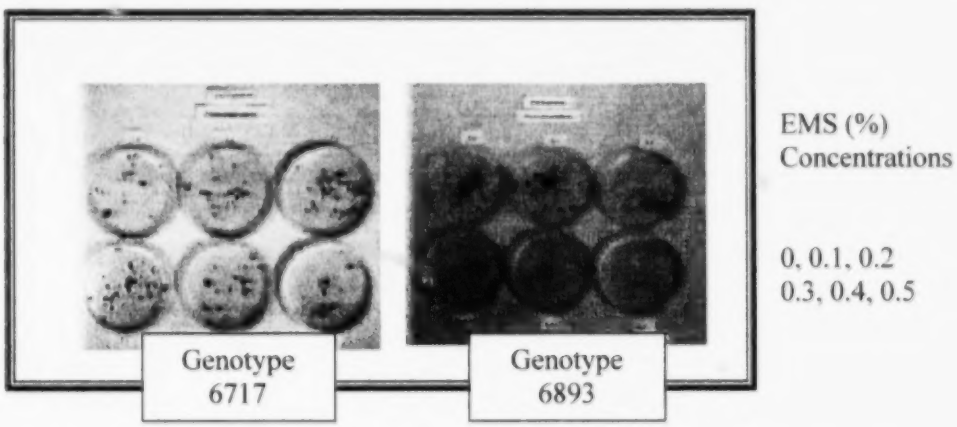


Figure 2. EMS-treated microspore derived embryos of *B. juncea* lines 6717 and 6893



d) Embryo development and plant regeneration

i) Basic protocol

Three weeks after microspore induction, the embryos are placed on a gyratory shaker at 22°C with a 16-hour photoperiod. After 5-7 days, the embryos change colour from pale yellow to green and then stored at 4° C for a minimum of two weeks before transfer to solid media. The embryos are then transferred to 100 X 15 mm plates containing B5 solid media with 1% agar and 1% sucrose (pH 5.8) for germination. As required, the

germinated plantlets are subcultured and transferred to large petri dishes (150 X 25 mm) containing B5 media with 0.8 % agar and 2 % sucrose (pH 5.8) for further development, before transferring to the greenhouse.

ii) Determination of regeneration frequency

Desiccation experiments were conducted using abscisic acid (ABA 10^{-4} M) to determine whether embryo development and regeneration could be enhanced. Embryos obtained from the initial experiments with the commercial cultivars were used. The ABA was applied directly to mature embryos in their original culture media and placed in the dark on a shaker for 7 days. The embryos were then plated on sterile filter paper and placed into desiccators. There was at least 20 % improvement in the regeneration frequencies of the genotypes tested (Table 3).

Table 3: Percent regeneration of desiccated embryos of several *B. juncea* varieties.

Genotype			
	Control (No ABA)	**Control (ABA)**	**Desiccated (ABA)**
Krisna	9%	54 %	51 %
Cutlass	74 %	95 %	97 %
Donskja	NA	66 %	88 %

Other experiments were also conducted to determine regeneration frequencies of the two *B. juncea* breeding lines by varying the length of cold treatments at 4 °C. As well, ABA was used in combination with desiccation of the embryos to determine the conversion of embryos to plants. The embryos were obtained from an experiment with EMS (BJ64) and were split into groups with some of the embryos receiving 10^{-4} M ABA and either desiccated or regenerated with no additional treatments. One set of controls received no treatments while the other set of controls were only desiccated with no additional treatments. The remaining embryos were given cold treatment for varying lengths of time from 14 days to 36 days.

Results indicated that those embryos receiving only an ABA treatment and those embryos which only received cold treatment for 21-36 days, regenerated into normal plantlets. While those embryos which were desiccated and received no cold treatment or ABA did poorly or did not survive (Table 4).

Table 4: Regeneration (%) of embryos of plant line 6717 after desiccation and cold treatment.

EMS Experiment BJ 64	Control (no cold)	Control (no cold or ABA) + desiccated	ABA-shaker for 7 days (no cold)	ABA and desiccated	Cold 4°C for 14 days	Cold 4°C for 21 days	Cold 4°C for 30-36 days
7.0	N/A	N/A	100	N/A	84	97	99
7.1	88	0	97	67	90	98	96
7.2	78	17	94	45	70	76	87

For ease of double haploid production, all embryos obtained from EMS experiments were stored at 4 °C for a minimum of two weeks before plating to B5 solid media (1% agar, 1% sucrose, pH 5.8). Cold treatments of the embryos for two weeks were sufficient to enhance embryo conversion to plantlet.

e). Plant development and seed production

i) Basic protocol

Plantlets were transferred directly from the tissue culture room into prepared jiffy peat pellets, after germination on B5 solid medium (1 % agar, 1% sucrose, pH 5.8). The plantlets were then placed into a growth cabinet (20/ 15°C, 16-hour photoperiod) and acclimatized for two weeks. Once the plantlets have a well-developed root and shoot system, they were transferred to individual 4" pots and transferred to the greenhouse.

The majority of mutagenized double haploid plants produced were normal and vigorous with good seed production. There were 29, 400 plantlets regenerated and just over 20, 000 plants were transferred to the greenhouse for further development. Of the 20,000 plants transferred to the greenhouse, 9100 produced seed.

Overall, genotype 6893 produced 3650 plants and genotype 6717 produced 5450 plants. The remaining plants either did not survive transplanting or were haploid and discarded or did not produce seed. Each double haploid plant was harvested separately once they reached maturity. The seeds weights ranged from 0.1 g to 6.5 g with the average seed weight of 0.5g for genotype 6893 and 0.8 g for genotype 6717.

ii) Plant production

Experiments were conducted using different soil mediums to determine the best medium for establishing a good root structure before transferring the plants to 4" pots. Sunshine soilless mix, Redi-earth/perlite mixture and jiffy peat pellets were compared. Visible differences were observed between treatments. The plants transferred to Sunshine mix showed highly visible signs of senescence while the Redi-earth mixture showed some signs of senescence. The plants transferred to jiffy peat pellets were healthy and green with an extensive root structure (Figure 3, 4 and 5).

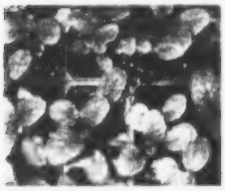


Figure: 3

Sunshine mix

Figure: 4

Redi-earth/perlite mixture

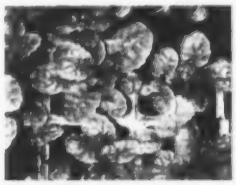


Figure: 5

Jiffy peat pellets

Plantlets were then transferred directly into 4" pots and acclimatized in the greenhouse. The dome covers were slowly removed and the plants were hand watered until the first flower buds appeared.

Results indicated that those plantlets transferred directly to jiffy peat pellets were healthy, normal and vigorous. This procedure was routinely incorporated into the double haploid plant production protocol.

iii) Biological controls and sulphur applications

Biological controls (*Amblyseius cucumeris*) are used in the greenhouse to keep the plants free from thrips, which pose as a threat to seed production of the double haploid plants. This mite is a safe alternative to using costly and toxic sprays in a greenhouse environment. *A. cucumeris* are shipped dormant at all life stages, mixed in with a food source, usually bran flakes, and applied directly onto the plants. For a slow release system, the mites are also shipped in paper sacks containing this same mixture, and are hung directly on maturing plants. The mites can live on the plants for up to 15 weeks, providing effective control of thrip populations.

Admire, a non-toxic systemic chemical was also used to control aphids infestations. This chemical was administered as required, mixed at the proper concentration and applied directly into the reservoir of the Ebb flow system.

B.juncea is highly susceptible to powdery mildew in a greenhouse environment. To remedy this situation, a Hotbox Sulfume was installed in each greenhouse which emits elemental sulphur at a continuous rate.

f. Field experiments

There were 330 double haploids planted in the field in collaboration with AAFC in the spring of 2002. One row each of the 330 lines was planted with alternating rows of AC Parkland (*B. rapa*) and AC Excel (*B. napus*) as checks. The parental lines TO92-223 (6893) and J00-6717 (6717) were also used as checks.

Some of the double haploid lines did show lots of branching and good pod density. The double haploids tended to be slightly taller than the parental line and checks (Table 5). On average, the double haploid lines were similar to the parental lines for days to flowering, however some lines were 5 days later. This was also shown in days to maturity as some of the *B. juncea* double haploids matured early (84-89 days) and some were very late (96-105 days). There were some missing rows as well as partial rows due to poor germination in the spring. The double haploids were also slightly affected by blister beetle damage during the summer months. A few of the double haploid lines showed some sterility.

Table 5. Comparison of *B. juncea* Double Haploid lines and Parental lines with *B. napus* and *B. rapa* checks under field conditions in 2002.

	Height (cm)		**Days to Flower**		
	Average	**Range**	**Average**	**Range**	**Days to Maturity**
AC Excel	91	80-110	47	45-48	Mid-Late
AC Parkland	79	65-110	39	38-39	Early
***B. juncea* double haploids**	121	70-130	44	41-48	Early-Very Late
TO92-223(6893)	100	85-115	43	43	Mid
J016717(6717)	90	90-100	43	43-44	Mid-Mid Late

Seed from 2200 double haploid lines were planted in 2003 field trials in collaboration with AAFC. One row each of the 2200 lines were planted with alternating rows of AC Parkland (*B. rapa*) and AC Excel (*B. napus*) as checks. The parental lines TO92-223 (6893) and J00-6717 (6717) were also used as checks. The double haploid lines were seeded May 2003 and although moisture conditions were good for seeding, there were several missing rows across the ranges, as well as partial rows due to poor germination. The double haploids were also affected by a flea beetle infestation after germination but were sprayed with a pesticide.

A field tour of the *B. juncea* double haploid plots at AAFC research farm was conducted in August 2003 with Dr. G. Rakow and staff. Many of the rows across the ranges had only a few plants, but there was still enough of each double haploid line to produce a sample for chemical analysis to determine the fatty acid profiles and levels of saturates. It was concluded that all rows would be harvested and analyzed.

Under field conditions, there were distinct differences between the two genotypes. The double haploid plants were similar to their parental lines; however, the double haploids were more vigorous and slightly taller in height. Days to maturity were from the mid to mid late range for the parental lines, while the double haploid lines ranged in maturity from early to very late. The following observations were made for each breeding line:

Breeding line 6893:

- Light green in colour
- Earlier maturity
- Tall in height
- Large pods (~1-1.5 inchs in length)
- Good fertility
- Good seed set, seeds packed tightly
- Many seeds in each pod (> 20)
- Constant number of seeds per pod
- No seed abortion

Breeding line 6717:

- Dark green in colour with more anthocyanin produced
- Later maturity
- Short in height
- Shorter pods (<1.5 inches)
- Good fertility
- Large seed, but not consistent seed set (<20)
- Early seed abortion
- More disease occurrence such as staghead and aster yellows

g. Fatty acid analysis

The 330 double haploid lines planted in 2002 field trials were analysed for fatty acid composition. The objective for this *B. juncea* mutagenesis project was to produce double haploid lines with less than 7.0 % total saturate levels. The parental lines J01-6717 (6717) and T092-223 (6893) total saturates were 6.74 and 6.61 respectively (Table 6). The variety checks AC Excel (*B. napus*) total saturate levels were 6.67 and AC Parkland (*B. rapa*) total saturate levels were 5.42. The total saturate levels for the double haploids ranged from 5.41-9.54 for genotype 6717 and from 6.13-9.05 for genotype 6893 (Table 6). There were six double haploid lines of interest from genotype 6717, with total saturates less than 6.0. Other variations in the fatty acid profile for genotype 6717 were high levels of stearic acid (3.76), reduced levels of oleic acid (17.19) and high levels of erucic acid (28.28).

None of the double haploid lines from genotype 6893 produced variants with total saturates less than 6.0 (Table 6). The total saturates ranged from 6.13 to 9.05. Some variations of interest for this genotype were lower levels of oleic acid (33.03) and elevated levels of erucic acid (8.97) when compared to the parental germplasm.

Table 6: Fatty acid analysis of *B. juncea* mutagenized double haploid lines, check varieties and parental material grown in 2002.

Fatty Acid *	AC Excel (*B. napus* check)	AC Parkland (*B. rapa* check)	J01-6717 (6717 parent)	T092-223 (6893 parent)	6717 DH Range (Average)	6893 DH Range (Average)
C12:0	0.001	0.004	0.004	0.007	0.000 - 0.010 (0.002)	0.000 - 0.012 (0.005)
C14:0	0.038	0.038	0.046	0.055	0.040-0.070 (0.054)	0.051 -0.100 (0.058)
C16:0	3.59	3.17	3.51	3.50	2.67-4.22 (3.32)	3.07-4.29 (3.50)
C18:0	1.89	1.37	2.22	2.01	1.14- 3.76 (1.90)	1.73-3.37 (2.00)
C18:1	61.85	55.26	54.14	42.74	17.19 - 59.16 (35.58)	33.03 - 46.75 (42.42)
C18:2	17.96	22.13	22.83	34.26	16.11-31.94 (21.54)	30.01 -38.02 (34.53)
C18:3	10.18	14.50	13.33	13.62	9.39- 17.12 (13.50)	10.25-15.93 (13.55)
C22:1	0.46	0.38	0.32	0.00	0.21- 28.28 (11.77)	0.02-8.97 (0.21)
Total saturates	6.67	5.41	6.74	6.61	5.40-9.54 (6.77)	6.13-9.05 (6.62)

*C12:0 =Lauric, C14:0 =Myristic, C16:0 =Palmitic, C18:0 =Stearic, C18:1 =Oleic, C18:2 =Linoleic, C18:3 = Linolenic, C22:1 =Erucic

4. Conclusion

Two breeding lines, 6717 and 6893 were used for microspore mutagenesis experiments. In addition, embryogenic donor plant lines and embryogenic double haploid plants of 6717 were self-pollinated and also used for microspore mutagenesis experiments. The embryogenic frequencies varied between the two breeding lines and the selfed populations of breeding lines 6717 that were evaluated.

There were 142 microspore mutagenesis experiments conducted using 0 (control), 0.1 and 0.2 % EMS. Experiments were conducted to improve efficiencies for the microspore culture system, embryo culture, plantlet regeneration and the greenhouse growing conditions. As a result, over 20,000 plants were produced and 9,100 were diploid and set seed. Of these double haploid lines produced, 330 were field grown in 2002 and there were 2200 grown in the field in 2003. Fatty acid analysis was completed for the 330 lines grown in 2002.

Results from 2002 field trials indicated that there were some lines that had lowered levels of saturated fatty acids compared to the parental lines. There are also other double haploid lines that may be of interest to screen for other desirable characteristics or traits. Fatty acid analysis for the double haploids grown in 2003 are expected to be completed in the early spring of 2004.

The protocol developed for microspore mutagenesis of *B. juncea* is described as follows:

a. Donor plant conditions

The donor seeds are planted in 6" pots filled with Redi-earth soil-less mix supplemented with Nutricote (14-14-14) slow-release fertilizer. Two to three seeds per pot are planted and then are thinned to one plant per pot at the 2-3 leaf stage of growth. The pots are placed in a Conviron growth chamber (20/15°C, 16 hour photoperiod) and watered three times a week with a fertilizer regime of 0.35 g/L of 15-15-18 (N-P-K) fertilizer. At approximately 3 weeks, when the first buds are observed on the plants, the temperature is lowered to 10/5°C. The plants are maintained with vigorous trimming of excess foliage and spent inflorescences to keep the plants compact and to produce a good supply of buds for microspore mutagenesis experiments.

b. Microspore culture

Approximately 30-50 buds, 2-4 mm in size, are selected and placed in Lipshaw baskets. Extra care is taken to select buds that are free from any discoloration or imperfections due to difficulties with the sterilization process. The baskets are surface sterilized in 30% ethanol for one-two minutes supplemented with 5ml of a 10% solution of tween (filter-sterilized). The lipshaw baskets are then removed from the ethanol solution and placed on sterile filter paper in a laminar flow hood and allowed to dry briefly and then lightly flamed by passing the baskets over a bunson burner. The baskets are then sterilized in full strength bleach (5.25 % sodium hypochlorite), supplemented with 5 ml of a 10% Tween (filter-sterilized) solution for 15 minutes on a gyratory shaker. After sterilization is complete the baskets are rinsed three times (5 minutes each) with sterile distilled water.

Microspores are isolated by macerating the buds with a glass rod in 5 ml of half-strength B5 medium [Gamborg, 1968] supplemented with 13 % sucrose and filtered through a 44 µm screencloth (nitex) into a sterile 50 ml tube. The beaker and filter are rinsed three times with 5 mL of B5-13 to bring the total volume in the centrifuge tube to 20 ml. The crude microspore suspension is centrifuged at 130-150g (1200 rpm) for three minutes and the supernatant is decanted and the pellet is then resuspended in 5 ml of B5-13. The pellet is washed twice more, repeating the centrifugation for a total of three washes with B5-13.

After the final centrifugation step, the supernatant is decanted and the pellet is resuspended in 10 ml of NLN-17 with no glutamine and 0.1 % BA. The EMS and microspore suspension are allocated to three sterile 50ml centrifuge tubes according to the table below:

EMS treatment	**Microspore suspension (ml)**	**Additional NLN-17 (ml) without glutamine+BA**	**1% EMS (ml)**
0 (control)	2	8	0
0.1%	4	5	1
0.2%	4	4	2
Total =	10	10	10

The microspores and EMS are incubated at room temperature (24°C) for 1.5 hours. During this time the tubes are gently mixed to keep the microspores in suspension. After the incubation period, the tubes are centrifuged for 3 minutes (130-150g) and the supernatant is discarded into a 5N solution of NaOH (20g/100ml H20. The microspores are washed with B5-13 as described above for a total of three additional washes and the supernatant is again discarded into the NaOH solution.

To make a 1% solution of EMS:

- In a laminar flow bench, 9.9 ml of NLN-17 without glutamine + BA are added sterilely to a 50ml centrifuge tube
- In a chemical flow hood, 0.1 ml of EMS is also added to the tube and mixed gently using a 10 cc syringe
- The 1% EMS solution is filter sterilized using a 10 cc syringe and 0.2 µm filter in a laminar flow bench into another sterile 50 ml centrifuge tube
- The solution is made up fresh for each mutagenesis experiment

c. Chromosome doubling

A hemocytometer is used to determine the number of microspores that are in suspension for each treatment. The required amount of NLN-17 without glutamine and with BA and colchicine (10^{-4} M) is added to each tube to obtain a density of

1.0 X 10^5 microspores/ml. Each treatment is then individually plated with ten ml of suspension dispensed into100x15mm sterile petri plates and the plates are sealed with parafilm.

d. Culture conditions

Depending on the genotype, the cultures are placed in either a 32°C or 35°C incubator and left in the dark for 48 hours or 72 hours. After the required amount of time, the media is changed to NLN-13 with glutamine to remove the colchicine from the cultures. The media is removed from the individual plates by using a pipette and dispensing into sterile 50 ml centrifuge tubes. The tubes are centrifuged for 3 minutes (130-150g) and the supernatant is poured off and the same amount of NLN-13 is added to each tube. Each treatment is re-dispensed back into the original plates, resealed and returned to a 24°C incubator for the remainder of three weeks. Visible embryos can be seen with the naked eye at 10-14 days after the induction.

e. Embryo culture

Three weeks after induction, the embryos are placed on a gyratory shaker at 22°C with a 16-hour photoperiod until they turn green, usually within 5-7 days. The plates of embryos are then counted and moved to 4°C for 2-3 weeks to enhance regeneration. The embryos are transferred to100x15 mm petri plates containing solid B5 media (1% agar, 1% sucrose, pH 5.8) and placed into a tissue culture room (22°C, 16 hour photoperiod) for 4-6 weeks to germinate into plantlets.

f. Plantlet culture

After 4-6 weeks, the normal plantlets are transferred to jiffy peat pellets, covered in trays, and acclimatized in growth cabinets at 20/15°C for two weeks. Depending on space and resources, the plantlets are sometimes transferred to150x25 mm petri dishes containing B5 solid media (0.8% agar, 2% sucrose, pH5.8) and returned to the tissue culture room. These plantlets can then be transferred to jiffy peat pellets in 4 weeks and acclimatized before they are transferred to the greenhouse.

g. Greenhouse growing conditions and harvest

After two weeks of acclimatization and once the plantlets have obtained well developed root and shoot systems, the plantlets are then transferred to individual 4" pots (15 per flat) containing Redi-earth soil-less mix and perilite supplemented with nutricote (14-14-14). The flats remain covered with dome lids to maintain the humidity while adjusting to greenhouse growing conditions (24°C, 16 hour photoperiod and supplemented with high pressure sodium lighting). The lids are removed and the plantlets are hand watered until flowering begins. Ebb flow benches are switched on and the plants are watered every two days. Nutrients are supplied with every watering with 0.35 g/L of 15-15-18 (N-P-K) fertilizer.

Once the plants begin to flower, usually within 4-6 weeks, the diploid plants are bagged and the haploid plants are discarded. Seed sets within 8-12 weeks, excess foliage is removed and the individual plants are secured to their stakes. For the next 2-3 weeks the plants are lightly watered until maturity. Seed is harvested from the individual plants,

weighed, and then the seed weights are recorded on Excel spreadsheets. The seed is stored in cold storage until required for field trials.

C. Personnel

The ADF grant provided funding for a full-time technical officer from December 11, 2000 to December 10, 2003.

D. Equipment

No equipment was purchased for this project. Growth cabinet space was rented from SOCO at Innovation Place.

E. Project developed material

There were 9100 double haploid lines of *B. juncea* produced and 330 evaluated for altered fatty acid profiles. This material can also be evaluated for other desirable characteristics to be incorporated into a canola breeding program.

F. Project photos

Photos are included in the final report.

G. Acknowledgements

A paper was presented at the 13th Crucifer Genetics Workshop in Davis, CA in March 2002 discussing the applications of double haploidy and mutagenesis in canola species of *B. rapa*, *B. napus* and *B. juncea*. (Abstract is attached). ADF was acknowledged in the presentation. In addition, when groups tour the facilities we discuss projects underway and we acknowledge ADF for their support.

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